INTERNATIONAL JOURNAL OF ONCOLOGY 5: 1249-1253, 1994

Genomic instability and LOH at two polymorphic sites in the H-*ras*1 gene

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Abstract. Several repetitive elements have been associated with and identified in the surrounding region and within the human c-H-*ras*1 gene. The polymorphism exhibited by these sites provides valuable information regarding the function and structure of the H-*ras* gene. We have investigated two such polymorphic sites: i) a hexanucleotide microsatellite region (HRMS) within intron 1 of H-*ras* and ii) the VTR region at the 3' end of the gene. Comparison between normal and tumor tissues from 25 samples of bladder cancer revealed that 5 samples (20%) exhibited LOH at these polymorphic sites indicating that an allelic loss had occured at the H-*ras* locus. Furthermore, instability was detected in 4 cases at the hexanucleotide locus, while the VTR region was found unaffected. The two polymorphic sites are in a strong linkage disequilibrium in normal tissues, while in tumor tissues with genomic instability this linkage is altered, possibly leading to differential regulation of the H-*ras*. Also a previously reported allele at the HRMS locus was found to behave in a manner that preserves the linkage between the two polymorphic sites in normal tissues.

Introduction

In the human c-H-*ras*1 gene and the surrounding regions, units of nucleotide sequences are tandemly repeated in several regions. One prominent polymorphic site is a result of a variable tandem repeat (VTR) of a unit sequence of 28 bp in the 3' region of the gene (1). Different numbers of the repeating unit give alleles of a wide range in size. Four alleles - denoted as a1, a2, a3 and a4 - are the most common accounted, with a frequency of 94% in the general population (2,3). These alleles have been divided into two subgroups: one comprising the small a1 and a2 alleles with approximately 40 copies of the 28 bp core sequence and a second one comprising the larger a3 and a4 alleles consisting of approximately 80 copies of the core sequence (2). It has been proposed that these alleles have served as progenitors for the remaining rare alleles (4). Previous studies have reported a significant association between the presence of the rare H-*ras*1 minisatellite alleles and an increased risk of common types of cancer (1 in 11 cancers of the breast, colorectum and bladder) (3). The tight linkage of this minisatellite to the H-*ras*1 locus has served as a marker to detect LOH involving the short arm of chromosome 11 in a variety of human cancers, such as bladder (5), breast (6), lung (7) and ovarian cancers (8).

Intron 1 of the c-H-*ras*1 gene has also been shown to posses a tandemly repeated sequence 83 bp upstream from the 5' end of exon 1 (HRMS) (9). This length polymorphism is the result of a repeated hexanucleotide sequence based on the consensus GGGCCT. The number of repetitions produces alleles with two (P2), three (P3) or four (P1) times the core hexanucleotide sequence (10), which show a linkage disequilibrium with the VTR polymorphism (9).

Genomic instability involving mainly expansion of mono, di or tri-nucleotide repeats has been associated with a variety of hereditary diseases (Huntington's disease, myotonic dystrophy, fragile X syndrome) as well as HNPCC (11-15). These length alterations have been shown to be the result of a mutator phenotype producing consecutive somatic slippage events of a single or a few repeated units. The gene(s) responsible for these events have been implicated in DNA repair mechanisms (16,17).

LOH has been shown to contribute to tumor development mainly by inactivating tumor suppressor genes. Recently it has been shown to affect chromosomal regions involving the c-H-*ras*1 gene (18). This suggests a more complex role by which *ras* genes are involved in carcinogenesis.

In this work we have examined the effect of genomic instability and LOH on the c-H-*ras*1 locus in 25 samples of bladder tumor. Data for these genomic aberrations were obtained from the two polymorphic sites: i) the VTR region and ii) the HRMS site present within the gene.

Materials and methods

DNA extraction. DNA was extracted from tissues by proteinase K and phenol/chloroform extraction as previously described (19).

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Key words: H-*ras*1, variable tandem repeat, microsatellite, loss of heterozygosity, microsatellite instability

Table I. The polymorphic status at the hexanucleotide repeat site (HRMS) and VTR site of the c-H-*ras* 1 gene in normal and tumor bladder tissues.

Sample no	HRMS site (normal tissues)[a]	HRMS site (tumor tissues)	VTR site (normal tissues)[b]	VTR site (tumor tissues)
1	P1/P1	na	a1/a1r	na
2	P1/P2	na	a1r/a4	na
3	P1/P3	P1/-(LOH)	a2r/a1	-/a1(LOH)
4	P1/P2	na	a1/a3	na
5	P1/P2	na	a1r/a4	na
6	P2/P2	P2/P1(HI)	a3/a3	na
7	P2/P2	P2/P1(HI)	a3/a4	na
8	P1/P1	na	a1/a1	na
9	P1/P2	-/P2(LOH)	a1/a4	-/a4(LOH)
10	P1/P3	P1/-(LOH)	a1/a1r	a1/-(LOH)
11	P1/P1	na	a1/a1r	a1/-(LOH)
12	P2/P3	na	a1r/a4	na
13	P2/P2	na	a3/a3	na
14	P1/P1	na	a1/a1	na
15	P1/P1	na	a1/a1r	na
16	P1/P1	na	a1/a1	na
17	P1/P3	na	a1/a2	na
18	P1/P2	-/P2(LOH)	a1/a4	-/a4(LOH)
19	P2/P2	na	a4/a4	na
20	P2/P2	na	a4/a4r	na
21	P2/P2	na	a4/a4	na
22	P1/P1	na	a1/a1r	na
23	P2/P2	P2/P3(HI)	a3/a3	na
24	P1/P1	P1/P2(HI)	a1/a1r	na
25	P3/P3	na	a2/a2	na

[a]P1=4 hexanucleotide repeat allele, P2=2 hexanucleotide repeat allele, P3=3 hexanucleotide repeat allele; [b]a1, a2=small common VTR alleles, a3, a4=large common VTR alleles, a1r, a2r, a3r, a4r= rare (small and large) VTR alleles. HI=hexanucleotide instability. LOH=loss of heterozygosity. na=not affected.

PCR analysis. Amplification of VTR H-*ras* alleles were performed in 50 µl reactions with: 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH=9.0 (25˚C), 0.01% (w/v) Tween 20, 2% (v/v) DMSO, 1.5 mM $MgCl_2$, 400 µM dNTPs, 1 µM each oligonucleotide primer, 200-400 ng of genomic DNA and 2.5 units of Taq polymerase (Advanced Biotechnologies). The amplimer sequences (20) were: 5'-GAGCTAGCAGGGCAT GCCGC-3' and 5'-AGCACGGTGTGGAAGGAGCC-3' Twenty-five cycles of denaturation for 1 min at 95˚C, annealing for 20 sec at 65˚C and elongation for 4 min at 72˚C were carried out in a DNA thermal cycler (480-Perkin Elmer Cetus).

VTR amplified products were resolved on 1.5% agarose gels (SeaKem-GTG), stained with ethidium bromide.

Amplification of HRMS alleles were performed in 50 µl reactions with: 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH=9.0 (25˚C), 0.01% (w/v) Tween 20, 200 µM dNTPs, 2.5 mM $MgCl_2$, 1 µM each oligonucleotide primer, 200-400 ng of genomic DNA and 1.25 units of Taq polymerase. The amplimer sequences (10) were: 5'-CTGTGGGTTTGCCCTT CAGA-3' and 5'-CTCCTACAGGGTCTCCTGCC-3'. Thirty cycles of denaturation for 1 min at 95˚C, annealing for 35 sec at 63˚C and elongation for 40 sec at 72˚C were carried out in a DNA thermal cycler.

HRMS amplified products were resolved on 6% polyacrylamide gels and visualised by ethidium bromide or silver staining.

Amplification of the three HRMS alleles results in PCR products of: P1=118 bp, P2=106 bp and P3=112 bp (10).

Results

Detection of genetic alterations at the HRMS site. A panel of 25 bladder samples consisting of paired tumor and adjacent normal tissue was analysed for polymorphism at the HRMS locus. The analysis revealead that 9 out of 25 (36%) samples were informative for this locus. The pattern comparison between tumor and adjacent normal tissue in these 9 samples, revealed LOH in 4 tumor samples. Furthermore, from the remaining non-informative cases 4 exhibited an altered allelotype pattern between tumor and normal counterparts of the tissue. In all cases a different microsatellite allele was present in the tumor pattern, implicating the generation of a new allele (Table I).

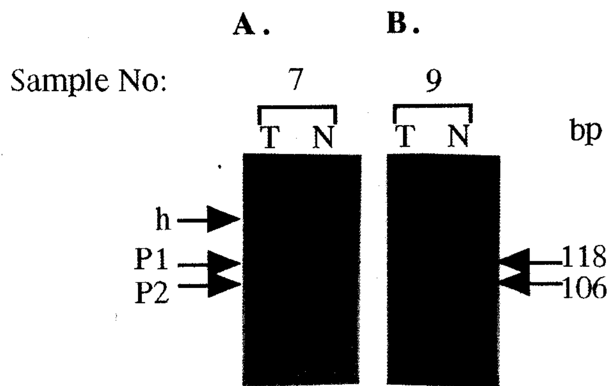

Figure 1. Representative results of microsatellite instability (A) and loss of heterozygosity (B) at the hexanucleotide region in bladder tissues (N=normal tissue, T=tumor tissue), (P1=4 hexanucleotide repeats, P2=2 hexanucleotide repeats, h=heteroduplex).

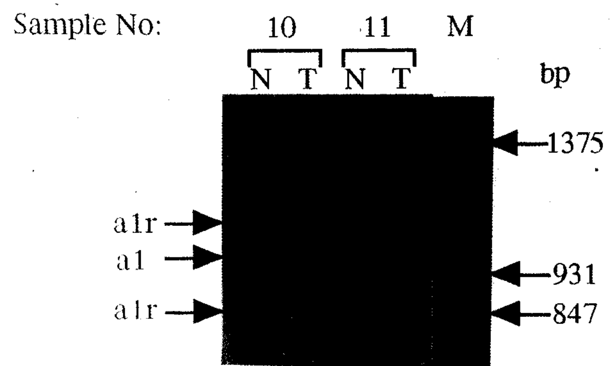

Figure 2. Representative results of loss of heterozygosity at the VTR region of H-*ras*1 in bladder tissues (N=normal tissue, T=tumor tissue, M=λ H+E marker), (a1=small common VTR allele, a1r= small rare VTR alleles).

Detection of genetic alterations at the VTR locus. Analysis at the VTR locus of the H-*ras* in the same panel of patients revealed that 16 out of 25 (64%) samples were informative for this polymorphic site. Comparison between tumor and adjacent normal tissue from the same patient in these samples showed that in 5 cases LOH had occured at this VTR locus. Moreover, 4 out of these 5 samples with LOH were the same that had shown LOH at the HRMS locus while the remaining sample with LOH at the VTR was non-informative at the hexanucleotide site (Table I).

Apart from the LOH detected in 5 samples at the VTR locus no other genetic alterations were observed, indicating that no minisatellite instability had occured at this site (Table I). A high frequency of rare VTR alleles was observed in these samples in tumor as well as normal tissues.

Correlation between HRMS and VTR polymorphism. It has been previously shown that a strong linkage disequilibrium exists between the alleles present at the HRMS and the VTR locus respectively (9). From the comparison of genotype patterns, present in the normal tissues, between the two polymorphic sites, we observed that this link is preserved. More specifically samples homozygous for alleles with two hexanucleotide repeats (P2) display large alleles (approximately 80 repeats of the core sequence) at the VTR locus. On the other hand samples homozygous for alleles with four hexanucleotide repeats (P1) are always linked with small VTR alleles (approximately 40 repeats of the core sequence). Samples that are heterozygous at the HRMS locus are also heterozygous for small and large VTR alleles. One interesting feature is the presence of a third hexanucleotide allele (P3) consisting of three repeat units with a frequency of 12%. This allele has been previously reported (10), but has not been linked with any of the VTR alleles. Samples 3, 10, 12, 17 and 25 in Table I demonstrate that this allele behaves like the four-hexanucleotide repeat allele (P1), that is always linked with small VTR alleles.

The tight linkage between the two polymorphic sites is well preserved in normal tissues and this is in good agreement with previous reports (9). An interesting feature is that this linkage is disturbed in tumor tissues exhibiting an altered linkage pattern. The four samples revealing genomic instability at the HRMS locus display different linkage patterns between normal and tumor tissues (Table I), not previously described.

Clinicopathological characteristics of the tumors presenting LOH and HRMS instability. Analysis of the clinicopathological characteristics of the tumors revealed that tumors with instability at the HRMS locus (samples 6, 24 and 7, 23) were Grade I and Grade II (respectively). They were small in size and presented no lymph node or metastasis incidence ($T_1N_0M_0$), suggesting a rather early stage of tumor development. In contrast four tumors (samples 3, 9, 8 and 10) with LOH were Grade III, except sample 18 which was Grade II, but of small size and no lymph node or metastasis occurence ($T_1N_0M_0$).

Discussion

The H-*ras*1 gene is involved in the mechanism of tumorigenesis in a variety of common cancers such as bladder, breast, ovarian, lung (21). These mechanisms include qualitative as well as quantitative abberations in the function or structure of the gene. Of these most common are point mutation activation, gene amplification and overexpression (21).

In this work we have examined structural modifications at polymorphic regions surrounding the H-*ras*1 locus, that can affect the integrity of this gene, in bladder tumors. Analysis of the two polymorphic sites revealed that five of 16 (31%) informative samples exhibited LOH. The data obtained was based especially on information from the 3' end VTR region of H-*ras* because of the heterozygosity (approximately 64%) exhibited by this site, compared to the hexanucleotide region which displays a heterozygosity of approximately 36%. Still this percentage is satisfactory in order to reconfirm the largest proportion of heterozygosity for the VTR locus.

Since the polymorphic sites are located upstream and downstream of the H-*ras* gene, this implies that in the five tumors with LOH an allelic loss has occurred for this gene. Such quantitative changes for the H-*ras* gene have been

previously described for other types of tumors such as breast and skin (6,18). Allelic imbalances which can lead to gene dosage effects have been implicated as alternative mechanisms by which *ras* genes are involved in the multistage tumor development (18). The frequency of this abberation (20%) in bladder tumors could be higher since the data obtained was based on information from heterozygous individuals, leaving the rest of the samples out of the analysis, despite the high degree of heterozygosity exhibited by the VTR region.

Allelic loss has been the main reason for the detection of loss of function of tumor suppressor genes. From this point of view allelic loss could mean inactivation of unknown tumor suppressor gene(s) located in the vicinity of H-*ras* gene on the short arm of chromosome 11. However, normal H-*ras* gene has been postulated to possess tumor suppressor function (22,23). This could indicate that H-*ras* has a more complex role in the development of cancer.

In this study instability was observed only at the hexanucleotide region where 4 out of 25 samples (16%) exhibited the generation of a new hexanucleotide allele. The VTR region was found unaffected by this proccess, which is in agreement with previously published results (13,14). This implies that instability affects microsatellite regions by different mutagenic mechanism(s) than minisatellite sites.

Two of the reported hexanucleotide alleles were found to be in linkage disequilibrium with VTR alleles (9). More specifically the large P1 allele (4 hexanucleotide repeats) is found linked to small alleles (common or rare) at the VTR locus. On the other hand the small P2 allele (2 hexanucleotide repeats) shows tight linkage to the large VTR alleles. A subsequent analysis at the hexanucleotide locus from a larger sample showed the existance of a third P3 allele consisting of 3 hexanucleotide repeats (10). The presence of this allele has not been previously correlated with any of the VTR alleles. In this study we have established that the P3 allele behaves in a similar fashion to the P1 allele in all normal tissues examined. This allele is always linked to small VTR alleles. Because of the small size of the population sample the analysis has to be expanded further to additional samples to establish the statistical significance of this linkage.

This allele combination between the two polymorphic sites has been proposed to reflect the results of the process of the human c-H-*ras*1 gene evolution (9). It is interesting to note that opposite combinations between alleles of the two polymorphic sites have never been observed, suggesting that such combinations may have some disadvantage for cell growth and that cells with these genes cannot survive (9). However in tumor tissues, like the ones examined in this work, due to: i) instability at the hexanucleotide locus resulting in generation of different alleles and ii) invariance at the VTR site, in some cases non-compatible combinations are created which do not exist in the surrounding normal tissue. In this connection it should be noted that enhancer activity of the VTR sequences has been reported (24). Moreover members of the rel/NF-κB transcription factors bind to H-*ras* VTR (25) and like other NF-κB binding sites VTR displays pleiotropic transcriptional regulatory activity that is promoter and cell type specific (20). Also the transforming activity of the H-*ras* gene as well as the levels of p21 depend highly upon the presence of the VTR region (4). On the other hand the hexanucleotide site lies in a conserved region of the H-*ras* intron1 that has been shown to possess a positive element which influences gene expression (26,27).

Taken together the data indicate that these polymorphic regions must play an important role in normal H-*ras* function. Furthermore, compatible allelic combinations between these sites probably ensure expression in normal tissues and function as safety valves against tumorigenesis as previously proposed (27). In tumor tissues due to instability of the hexanucleotide locus non-compatible combinations are created in the H-*ras* gene leading to a differential regulation of the H-*ras* alleles. Such a differential regulation may be one of the causes responsible for H-*ras* involvement in tumor development. This type of analysis should be extended to larger sets of samples in order to establish the statistical significance of this proccess which can represent an indirect activation of H-*ras* due to the mutator mechanism affecting microsatellite instability.

References

1. Capon DJ, Chen EY, Levinson AD, Seeburg PH and Goeddel DV: Complete nucleotide sequence of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302: 33-37, 1983.
2. Kasperczyk A, DiMartino NA and Krontiris TG: Minisatellite allele diversification: the origin of rare alleles at the H-*ras*1 locus. Am J Hum Genet 47: 854-859, 1990.
3. Krontiris TG, Devlin B, Karp DD, Robert NJ and Risch N: An association between the risk of cancer and mutations in the H-*ras*1 minisatellite locus. N Engl J Med 329: 517-523, 1993.
4. Krontiris TG, DiMartino NA, Colb M and Parkinson DR: Unique allelic restriction fragments of the human Ha-*ras* locus in leukocyte and tumor DNAs of cancer patients. Nature 313: 369-373, 1985.
5. Fearon ER, Feinberg AP, Hamilton SH and Vogelstein B: Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature 318: 377-380, 1985.
6. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J and Callahan R: Loss of a c-H-*ras*1 allele and aggressive human primary breast carcinomas. Cancer Res 46: 4776-4781, 1986.
7. Vachtenheim J, Horàkovà I and Novotna H: Hypomethylation of CCGG sites in the 3' region of H-*ras* protooncogene is frequent and is associated with H-*ras* allele loss in non-small cell lung cancer. Cancer Res 54: 1145-1148, 1994.
8. Lee JH, Kavanagh JJ, Wharton JT, Wildrick DM and Blick M: Allele loss at the c-Ha-*ras*1 locus in human ovarian cancer. Cancer Res 49: 1220-1222, 1989.
9. Iwahana H, Orita M, Kanazawa H, Hayashi K and Sekiya T: A new RFLP in intron 1 of the human c-Ha-*ras*1 gene and its close relationship with the variable tandem repeats in the region 3' to the gene. Oncogene 5: 1049-1053, 1990.
10. Tanci P, Genuardi M, Santini SA and Neri G: PCR detection of an insertion/deletion polymorphism in intron 1 of the H-*ras*1 locus. Nucleic Acids Res 20: 1157, 1992.
11. Caskey TC, Pizzuti A, Fu Y-H, Fenwick Jr RG and Nelson DL: Triplet repeat mutations in human disease. Science 256: 784-789, 1992.
12. Richards RI and Sutherland GR: Dynamic mutations: a new class of mutations causing human disease. Cell 70: 709-712, 1992.
13. Aaltonen LA, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B and de la Chapelle A: Clues to the pathogenesis of familial colorectal cancer. Science 260: 812-816, 1993.
14. Thibodeau SN, Bren G and Schaid D: Microsatellite instability in cancer of the proximal colon. Science 260: 816-819, 1993.

15. Shibata D, Peinado MA, Ionov Y, Malkhosyan S and Perucho M: Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. Nature Genet 6: 273-281, 1994.
16. Fishel R, Lescoe MK, Rao MRS, Copeland NG, Jenkins NA, Garber J, Kane M and Kolodner R: The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 75: 1027-1036, 1993.
17. Leach FS, Nicolaides NC, Papadopoulos N *et al*: Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 75: 1215-1225, 1993.
18. Bremner R and Balmain A: Genetic changes in skin tumor progression: correlation between presence of a mutant *ras* gene and loss of heterozygosity on mouse chromosome 7. Cell 61: 407-417, 1990.
19. Sambrook J, Fritsch EF and Maniatis T: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press NY, 1989.
20. Green M and Krontiris TG: Allelic variation of reporter gene activation by the H-*ras*1 minisatellite. Genomics 17: 429-434, 1993.
21. Field JK and Spandidos DA: The role of *ras* and *myc* oncogenes in human solid tumors and their relevance in diagnosis and prognosis. Anticancer Res 10: 1-22, 1990.
22. Spandidos DA and Wilkie NM: The normal human H-*ras*1 gene can act as an oncosuppressor. Br J Cancer 58: 67-71, 1988.
23. Spandidos DA, Frame M and Wilkie NM: Expression of the normal H-*ras*1 gene can suppress the transformed and tumorigenic phenotypes induced by mutant *ras* genes. Anticancer Res 10: 1543-1554, 1990.
24. Spandidos DA and Holmes L: Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras*1 gene. FEBS Lett 218: 41-46, 1987.
25 Trepicchio WL and Krontiris TG: Members of the rel/NF-κB family of transcriptional regulatory factors bind the H-*ras*1 minisatellite DNA sequence. Nucleic Acids Res 20: 2427-2434, 1992.
26. Honkawa H, Masahashi W, Hashimoto S and Hashimoto-Gotoh T: Identification of the principal promoter sequence of the c-H-*ras* transforming oncogene: deletion analysis of the 5'-flanking region by focus formation assay. Mol Cell Biol 7: 2933-2940, 1987.
27. Hashimoto-Gotoh T, Kikuno R, Takahashi M and Honkawa H: Possible role of the first intron of c-H-*ras* in gene expression: anti-cancer elements in oncogenes. Anticancer Res 8: 851-860, 1988.